

WHAT IS CLAIMED IS:

1. A method for examining nucleotide sequences which comprises:

(1) adding a group of primers consisting of multiple primer species to a solution containing a sample subjected to examination, and performing simultaneous synthesis of complementary strands at each of the multiple regions containing target nucleotide sequences to be examined;

(2) designing DNA probes with specific sequences so that elongation of complementary strands is affected by the presence or absence of mutations in said target nucleotide sequences wherein the same number of such DNA probes and said target sequences is used for elongation of complementary strands;

(3) performing elongation reaction of complementary strands using said targets or the sequences complementary to said targets as a template and the following reaction, in which pyrophosphate produced during said elongation reaction is converted to ATP and reacted with chemiluminescent substrates to develop luminescence, in the subcells of the reaction vessel that are compartmentalized for each said target; and

(4) detecting said luminescence, mutations present in said target nucleotide sequences.

2. A method for examining nucleotide sequences according to claim 1 which comprises:

(1) a group of said primers consisting of the

first anchor primers having an arbitrary first anchor sequence at the 5' terminus that is not hybridized with said targets;

(2) removing excess of said primers after said synthesis of complementary strands, followed by using a group of the second anchor primers for synthesis of complementary strands; and

(3) these second anchor primers containing an arbitrary second anchor sequence at the 5' terminus that is not hybridized with said targets and the sequence complementary to a part of the complementary strands synthesized by said complementary strand synthesis;

(4) preparing DNA strands with said first or second anchor sequence at least at one terminus by using these second anchor primers; and

(5) amplifying the number of copies of said DNA strands with said first or second anchor sequence as a priming region.

3. A method for examining nucleotide sequences according to claim 1 which comprises:

(1) said DNA probes are hybridized with said targets so that the 3' terminus of said primers is located exactly on said mutation sites by using single strands prepared from said amplified DNA strands as a template; and

(2) the elongation of complementary strands of said DNA probes depends on the base species at the

putative mutation site.

4. A method for examining nucleotide sequences according to claim 1 which comprises:

(1) a group of said primers consists of the anchor primers having an arbitrary anchor sequence that is not hybridized with said targets at the 5' terminus;

(2) each of the anchor primers are designed so that said mutation is located at the expected site when the 3'-terminal region is hybridized to said targets; and

(3) the elongation of complementary strands for each of said anchor primers is regulated by the presence of said mutations.

5. A method for examining nucleotide sequences according to claim 4 in which sequences of said anchor primers are varied so that it is easy to distinguish the correspondence between each of said primers and the sequence of said targets and that between the 3'-terminal region and the DNA of a mutant or a wild type.

6. A method for examining nucleotide sequences according to claim 1 in which a group of said anchor primers consists of the anchor primers having the first arbitrary anchor sequence at the 5' terminus that is not hybridized with said targets, excess of said primers is removed after said synthesis of complementary strands, and the second anchor primer, in which the 5'-terminal region contains an arbitrary second anchor sequence that is not hybridized with said

targets, the remaining region contains the sequence complementary to a part of complementary strands produced by said synthesis of complementary strands, and the 3'-terminal region is designed to be capable of hybridizing with said target sites of possible mutation, is used for the synthesis of complementary strands, the DNA strands thus obtained are used for synthesis of complementary strands by using the primers that are hybridized with either the complementary sequences of said first anchor or said second anchor sequences, and the copy number of DNA strands is amplified by either PCR amplification, rolling-cycle amplification or amplification using a loop structure.

7. A method for examining nucleotide sequences according to claim 1 in which said DNA probes contain the sequences specific to said target species and are immobilized to said subcells, and said amplified DNA strands are degenerated to prepare single strands, which are captured in said subcells by hybridization between this single-strand DNA and said DNA probes.

8. A method for examining nucleotide sequences according to claim 1 in which said DNA probes contain the sequences specific to said target species and are maintained with matrix in said subcells, and said amplified DNA strands are degenerated to prepare single strands, which are captured in said subcells by hybridization between this single-strand DNA and said DNA probes.

9. A method for examining nucleotide sequences according to claim 1 in which said DNA probes contain the sequences specific to said target species and are maintained in said subcells, said amplified DNA strands are degenerated to prepare single strands, which are captured in said subcells by hybridization between this single-strand DNA and said DNA probes, and synthesis of complementary strands is performed with the circular DNA added to said subcell as a template by using the anchor sequence containing the 3' terminus of said single-strand DNA thus captured or of their complementary strands as a primer sequence.

10. A method for examining nucleotide sequences according to claim 9 in which the primers used for synthesis of complementary strands in each of said subcells are common to said multiple subcells.

11. A method for examining nucleotide sequences according to claim 1 in which the anchor sequence containing the 3'-terminal region of said DNA strands captured in said subcells or their complementary strands is used as a primer to perform synthesis of complementary strands using the DNA with a loop structure separately added as a template.

12. A method for examining nucleotide sequences according to claim 9 in which the capture of DNA strands subjected to complementary strand synthesis is achieved by binding of said DNA strands to said DNA probes immobilized to the solid surface of said

subcells or to the surface of beads placed in said subcells.

13. A method for examining nucleotide sequences according to claim 1 in which pyrophosphate is produced during synthesis of complementary DNA strands containing the sequence that is not related to said target DNA.

14. A method for examining nucleotide sequences according to claim 1 in which the probes used for preparing the first complementary strands from a sample are the first anchor primers, whose anchor region is self-hybridized to form a loop-like complementary strand, which acts as templates for the second synthesis of complementary strands in said subcells by using the second anchor primers and the third primers, and pyrophosphate produced by formation of loop-like DNA strands in said second synthesis of complementary strands is converted to ATP, which is consequently utilized for chemiluminescent reaction.

15. A method for examining nucleotide sequences characterized by detection of mutations in genes or DNA with the following steps:

(1) a group of probes consisting of multiple probe species is added to a sample solution subjected to examination;

(2) two said probes are hybridized with each of the different target sequences;

(3) the DNA probes are prepared so that the

binding reaction of two said probes in ligation is affected by the presence of base mutations in DNA subjected to examination;

(4) using a group of said probes consisting of pairs of these probes, long DNA strands are prepared by ligation reaction of two said probes;

(5) synthesis of complementary strands is performed at least once in subcells of a reaction vessel by using either said DNA strand or their complementary strand as a template; and

(6) pyrophosphate, the product of complementary DNA synthesis in each of said subcells, is converted to ATP and reacted with chemiluminescent substrates to develop luminescence in said subcells compartmentalized for each of said target sequences.

16. A method for examining nucleotide sequences according to claim 15 in which:

(1) each of said paired primers used in ligation used in ligation possesses the anchor sequence that is not hybridized to said target sequences at the 3' or 5' terminus;

(2) using said anchor sequences or their complementary strands, ligation products are PCR-amplified with said anchor sequences as a priming region; and

(3) all the subject DNA sites are simultaneously amplified and used as a template for synthetic reaction of complementary strands for



sequence containing the 3' terminus of said single-strand DNA thus captured in each said subcell or of their complementary strands as a primer sequence, synthesis of complementary strands is performed with the circular DNA added to said subcell as a template.

20. A method for examining nucleotide sequences according to claim 19 in which the primers used for synthesis of complementary strands in each of said subcells are common to at least said different subcells.

21. A method for examining nucleotide sequences according to claim 15 in which the anchor sequences containing 3'-terminal region of said DNA strands captured in said subcells or their complementary strands are used as a primer to perform synthesis of complementary strands using the DNA with a loop structure separately added as a template.

22. A method for examining nucleotide sequences according to claim 19 in which the capture of said DNA strands subjected to synthesis of complementary strands is achieved by the DNA probes immobilized to the solid surface of said subcells or to the surface of beads placed in said subcells.

23. A method for examining nucleotide sequences according to claim 15 in which pyrophosphate is produced during synthesis of complementary DNA strands containing the sequence that is not related to said target sequences.

24. A method for examining nucleotide sequences according to claim 15 in which:

(1) a pair of probes used for preparing the ligation product from a sample are the anchor primers, whose anchor region is self-hybridized to form a loop-like complementary strand;

(2) the product DNA of said ligation by a pair of said probes acts as a template for synthesis of complementary strands using the third primers in subcells; and

(3) pyrophosphate is produced during formation of loop-like DNA strands in synthesis of complementary strands.

25. A method for examining nucleotide sequences in which:

(1) said primers with a common sequence at their 5' termini can regulate synthetic reaction of complementary strands, where the target DNA strands are used as a template, depending on the presence or absence of mutations;

(2) following the complementary strand synthesis using said primers, pyrophosphate is produced during synthesis of complementary strands using either the DNA strands obtained by amplification of the product of said synthesis of complementary strands or their complementary strands;

(3) pyrophosphate is converted to ATP, which is subsequently used for chemiluminescence; and

(4) the presence of DNA mutations or the presence of target DNA is determined by luminescent intensity.

26. A method for examining nucleotide sequences characterized by detection of specific sequences and mutations in base sequences of said target DNA and by the following steps, using genomes or multiple target DNA as a template in which:

(1) multiple species of the first probes are hybridized to said templates in a single reaction vessel to prepare multiple species of the first complementary strands by the first synthesis of complementary strands;

(2) excess of the first probes are isolated and removed from said first complementary strands;

(3) with said first complementary strands as a template, the second synthesis of complementary strands is performed using multiple species of the second probes to obtain the second complementary strands, which partially contains the same sequence as that of said target DNA;

(4) in each compartmentalized area sorted with species of said first complementary strands, pyrophosphate is produced in the synthesis of said second complementary strands or in the complementary strand synthesis using said second complementary strands as a template, and is converted to ATP, which develops chemiluminescence for detection.

ABSTRACT OF THE DISCLOSURE

An effective method for examining nucleotide sequences of a sample having multiple test sites based on a method using chemiluminescence, which comprises a step in which a group of primer 1 consisting of multiple primer species is added to a solution containing a sample 2 subjected to examination, and simultaneous synthesis of complementary strands is performed at each of the multiple regions containing target nucleotide sequences to be examined; a step in which the DNA probes with specific sequences are designed so that elongation of complementary strands is affected by the presence or absence of mutations in the target nucleotide sequences wherein the same number of such DNA probes and the target sequences is used for complementary strand synthesis, 5-1 and 5-2; a step in which the elongation reaction of complementary strands using the targets or the sequence complementary to the targets as a template and the following reaction where pyrophosphate produced during the elongation reaction is converted to ATP and reacted with chemiluminescent substrates to develop luminescence are performed in the subcells of the reaction vessel that are compartmentalized for each target; wherein a step in which mutations present in the target nucleotide sequences are detected by detecting the luminescence. According to the method, sensitivity is greatly increased by amplification of the amount of pyrophosphate produced

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in synthesis of complementary strands without amplifying the copy number of targets.

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